Improved Culture Method for the Isolation of *Histoplasma capsulatum* and *Blastomyces dermatitidis* from Contaminated Specimens

COY D. SMITH, DR. P.H., AND NORMAN L. GOODMAN, PH.D.

Mycology Program, Department of Community Medicine, University of Kentucky Medical Center, Lexington, Kentucky 40506

ABSTRACT

Smith, Coy D., and Goodman, Norman L.: Improved culture method for the isolation of *Histoplasma capsulatum* and *Blastomyces dermatitidis* from contaminated specimens. Am. J. Clin. Pathol. 63: 276–280, 1975. Studies were performed to evaluate a method for selective isolation of *Histoplasma capsulatum* and *Blastomyces dermatitidis* from contaminated specimens. Ammonium hydroxide placed on an agar medium surface was found to inhibit the growth of many bacteria, yeasts, and saphrophytic fungi normally found in specimens such as animal tissues and sputum. In one study involving the culture of *B. dermatitidis* from canine tissues, 24% more isolations were obtained on a medium using NH_4OH compared with a similar medium. Increases in the isolation of *H. capsulatum* from sputum specimens were also obtained, ranging from 20 to 32% compared with four other media without NH_4OH. (Key words: *Histoplasma capsulatum; Blastomyces dermatitidis;* culture; Isolation; Selective culture; Ammonium hydroxide; Inhibition.)

IN KENTUCKY, as well as a major portion of the rest of this country, histoplasmosis and blastomycosis are common diseases in man and animals. Culture of the fungus from body exudates or tissue is the most reliable method to determine the etiology of disease. Specimens frequently contain a wide variety of other more rapidly growing saprophytic organisms (bacteria and fungi) that make the isolation of pathogenic fungi very difficult. Mycologists have made little progress in the improvement of isolation technics in the past 20 years. A major advance in technic was the development of a selective medium for the isolation of *Coccidioides immitis,* using antibiotics, *e.g.,* cycloheximide, penicillin, and streptomycin.[2] Cycloheximide inhibits or retards the growth of many fungal saprophytes, and penicillin and streptomycin help to control the bacteria. The use of these agents or other antibiotics has been adapted to use in media for the isolation of other systemic fungi and the dermatophytes. Even with the aid of antibiotics, the isolation of pathogenic fungi from proven cases is difficult.[4] One of the contributing factors, perhaps the most important, is overgrowth by saprophytes of the culture media. These organisms consist of antibiotic-resistant bacteria, yeasts, and

Received May 29, 1974; received revised manuscript August 9, 1974, accepted for publication August 9, 1974.

Address Reprint requests to Dr. Smith.

saprophytic fungi; however, culture plates do not have to be overgrown to inhibit growth of pathogens. As few as 10 colonies of *Candida albicans*, or other yeasts, on a plate can completely inhibit *Histoplasma capsulatum.*[3] The same holds true of many bacteria and fungi that produce toxins, antibiotics, and other inhibitory byproducts. Our experience in culturing specimens such as sputum for pathogenic fungi, using various media and combinations of antibiotics, has been that more than half the plates contain at least 1+ growth of saprophytes and a third contain 3+ or more growth.

This paper describes a new technic that reduces the growth of saprophytes from contaminated specimens, resulting in an increase in isolations of *H. capsulatum* and *Blastomyces dermatitidis.*

Materials and Methods

Three standard culture media were used in this study, brain–heart infusion agar containing 5% sheep blood; modified Sabouraud's dextrose agar,[1] and yeast extract–phosphate agar.[5] A simple method of preparing the stock solution of phosphate is to dissolve 40.0 Gm. of Na_2HPO_4 in 300 ml. of distilled water, then add 60.0 Gm. of KH_2PO_4. The pH is usually approximately 6.0. If necessary, add 1 N HCl or NaOH to obtain this value. Adjust the volume with distilled water to 400 ml. and store at 4 C. Two milliliters of the concentrated phosphate buffer are added to each liter of yeast extract medium to give a concentration of approximately 0.5 mg. phosphate per ml. of medium.

The above three media contain 2% agar, 50 μg. per ml. chloramphenicol, and 0.5 mg. per ml. cycloheximide. The experimental test medium was the yeast extract–phosphate medium with cycloheximide omitted. The specimens were cultured by spreading 0.5–1.0 ml. on each of the media in 15 × 100 mm. plastic petri dishes. Immediately, one drop (approximately 0.05 ml.) of concentrated NH_4OH from a sterile 1.0-ml. plastic pipette was dropped on the agar surface (off center). The ammonia was not spread but was allowed to diffuse throughout the media. All cultures were incubated at room temperature (22–25 C.) for as long as 4 weeks prior to the final reading out.

Table 1. Comparison of Two Yeast Extract Media for Isolation of *B. dermatitidis* from Canine Tissues

		YX(NH_4OH)*		
		Positive	Negative	Total
YX(A)†	+	35	3	38
	−	12	0	12
		47	3	50

* Contains chloramphenicol.
† Contains cycloheximide and chloramphenicol.

The first experiment was performed on cultures of dog tissues from animals experimentally infected with *B. dermatitidis.* The dogs were sacrificed, necropsied, and various tissues removed for culture; however, strict aseptic technics were not used. One milliliter of an approximate 1:10 dilution of the homogenized tissue (using mortar, pestle, sea sand, and sterile physiologic saline solution) was placed on each plate as the inoculum. Two plates of yeast extract medium, as previously described, were used, compared with two plates of yeast extract with NH_4OH.

The second experiment was a comparison of cultures of 160 sputum specimens, of which some were collected fresh and some were mailed in, from patients suspected of having histoplasmosis or blastomycosis. Each specimen was cultured in a similar manner on the three standard media and on the test medium with NH_4OH added.

A third trial evaluating the yeast extract medium with 0.05 ml NH_4OH per plate was performed on 1,762 sputum speci-

Table 2. Comparison of Three Media for Isolation of *H. capsulatum* or *B. dermatitidis* from 25 Positive Sputum Specimens*

Blood Agar†	Sabouraud's Agar†	YX (NH_4OH)‡		
		Positive	Negative	Total
+	+	9	0	9
+	−	5	1	6
−	+	3	1	4
−	−	6	0	6
		23	2	25

* 22 *H. capsulatum* and 3 *B. Dermatitidis.*
† Contains cycloheximide and chloramphenicol.
‡ Contains chloramphenicol.

Table 3. Comparison of Two Yeast Extract Media for Isolation of *H. capsulatum* and *B. dermatitidis* from 25 Positive Sputum Specimens

		YX(NH_4OH)*		
		Positive	Negative	Total
YX(A)†	+	18	1	19
	−	5	1‡	6
		23	2	25

* Contains chloramphenicol.
† Contains cycloheximide and chloramphenicol.
‡ Obtained using blood agar (Table 2).

mens cultured for pathogenic fungi in a diagnostic mycology laboratory. The three standard media described above were also used, but 20 units per ml. penicillin and 40 μg. per ml. streptomycin were used instead of chloramphenicol. In addition, yeast extract medium with 8 μg. per ml. gentamicin without cycloheximide was used.

Results

Of 50 tissues cultured for *B. dermatitidis*, 47 were positive (Table 1) using the medium with NH_4OH, compared with 38 of 50 on a similar medium with cycloheximide, but without NH_4OH. Only three isolations of the fungus were missed using NH_4OH on the medium.

From 160 sputum specimens, 25 isolations of *H. capsulatum* or *B. dermatitidis* were obtained using all the methods. The yeast extract medium with NH_4OH was superior for isolation compared with the blood agar and Sabouraud's agar, with 23, 15 and 13 isolations respectively (Table 2). The medium with NH_4OH missed only one isolation obtained with blood agar and one obtained with Sabouraud's agar, whereas blood agar missed nine and Sabouraud's agar missed 11. A comparison of the two yeast extract media on the same specimens showed that the medium with NH_4OH produced four more isolations than the one with cycloheximide (Table 3).

A total of 41 isolations of pathogenic fungi was made from the 1,762 specimens: 39 *H. capsulatum*, one *B. dermatitidis*, and one *Monosporium apiospermum* (Table 4). Again, the medium with NH_4OH was superior, with a 92% recovery rate of the total positives. The other two yeast extract media were second best, with 60% on the one with gentamicin and 58% on the one containing cycloheximide. Blood medium was almost equal, with 50% isolations, but Sabouraud's agar medium grew only 29%.

Discussion

Tests have shown the pH of the yeast extract medium to increase to 9.0–9.5 on the first day after addition of NH_4OH to the agar surface. The second day the pH falls to about 7.5, and the third day, to 6.5–6.8. These tests were performed by adding 5.0 ml. of fresh distilled water to the agar medium surface. The probes of a pH meter was inserted into the agar and the average of three readings recorded.

Preliminary results indicate the NH_4OH can also be used with blood agar and Sabouraud's agar, although it is not as effective on a rich medium. Cyclohex-

tions of the fungus were missed
OH on the medium.
50 sputum specimens, 25 isola-
I. capsulatum or *B. dermatitidis*
ned using all the methods. The
ict medium with NH_4OH was
or isolation compared with the
and Sabouraud's agar, with 23,
isolations respectively (Table 2).
um with NH_4OH missed only
on obtained with blood agar
btained with Sabouraud's agar,
lood agar missed nine and
's agar missed 11. A compari-
two yeast extract media on the
mens showed that the medium
)H produced four more isola-
the one with cycloheximide

of 41 isolations of pathogenic
made from the 1,762 speci-
I. capsulatum, one *B. dermatitidis,*
lonosporium apiospermum (Table
the medium with NH_4OH was
ith a 92% recovery rate of the
es. The other two yeast extract
second best, with 60% on the
entamicin and 58% on the one
cycloheximide. Blood medium
equal, with 50% isolations, but
's agar medium grew only 29%.

Discussion

ve shown the pH of the yeast
lium to increase to 9.0–9.5 on
y after addition of NH_4OH to
rface. The second day the pH
ut 7.5, and the third day, to
hese tests were performed by
ml. of fresh distilled water to
dium surface. The probes of a
vas inserted into the agar and
of three readings recorded.
ry results indicate the NH_4OH
e used with blood agar and
s agar, although it is not as
n a rich medium. Cyclohex-

imide was omitted when it was learned that the initial alkalinity produced by the NH_4OH reduced some of the activity of this antibiotic but had no obvious effect on chloramphenicol or on media with penicillin or streptomycin. The primary effect of NH_4OH appears to inhibit the growth of many bacteria and yeasts and, to a lesser extent, saprophytic fungi. Larger amounts of NH_4OH will affect the growth of *H. capsulatum* and *B. dermatitidis,* especially when the fungi are in the yeast phase, as in clinical specimens. The NH_4OH stock solution was maintained in screwcapped bottles at 4 C. Other pathogenic fungi, especially *Coccidioides immitis* and some of the dermatophytes, also appear to be resistant to NH_4OH. Therefore, the use of this compound may be of value for the isolation of these fungi as well.

The mechanism of selective inhibition of certain organisms by NH_4OH is not known. It does appear to have more effect on organisms that germinate more rapidly. Perhaps the NH_3 is affecting the organisms at a time when they are the most vulnerable, e.g., organisms that germinate within the first 12 hours prior to the loss of most of the NH_3 by volatilization. Trials using yeast extract–phosphate medium, without NH_4OH and cycloheximide, for sputum and animal tissue cultures resulted in overgrowth of plates by saprophytic fungi, in most cases with a low yield of pathogenic fungi isolations. Therefore, cycloheximide is a necessity when NH_4OH is omitted.

Conclusion

Yeast extract–phosphate medium with chloramphenicol and ammonium hydroxide was shown to be useful for the isolation of *H. capsulatum* and *B. dermatitidis* from contaminated specimens. From 50 dog tissues infected with *B. dermatitidis,* 47 isolations were obtained using this medium, compared with 38 using a similar medium with the addition of cycloheximide but omitting NH_4OH. One hundred and sixty (160) sputum specimens from patients suspected of having histoplasmosis or blastomycosis were cultured on brain–heart infusion agar with 5% blood, modified Sabouraud's agar, and yeast extract–phosphate agar media plates. These media contained chloramphenicol and cycloheximide. In addition, yeast extract–phosphate medium with chloramphenicol and NH_4OH was used. Twenty-five isolations (22 *H. capsulatum* and three *B. dermatitidis*) were obtained using the four media. The best medium was yeast extract–phosphate medium with NH_4OH, with which 23 isolations were obtained, compared with 19 on yeast-extract–phosphate medium with cycloheximide, 15 on blood agar, and 13 on Sabouraud's agar. A third trial using yeast extract–phosphate medium with NH_4OH and chloramphenicol, blood agar, Sabouraud's agar, and yeast extract–phosphate media as above, but substituting penicillin and streptomycin for chloramphenicol was compared. In addition, yeast extract–phosphate with gentamicin but without cycloheximide was used. From 1,762 sputum specimens, 41

Table 4. Recovery of 41 Pathogenic Fungi* from Sputum Specimens on Five Media

	Fungi Isolated	
	Number	%
Blood-A†	21	50
Sabouraud's-A†	12	29
Yeast Extract-A†	24	58
Yeast Extract-G‡	25	60
Yeast extract-NH_3§	38	92

* Consists of 39 *Histoplasma capsulatum,* one *Blastomyces dermatitidis,* and one *Monosporium apiospermum.*
† Contains cycloheximide, penicillin, and streptomycin.
‡ Contains gentamicin.
§ Contains chloramphenicol.

isolations of pathogenic fungi were obtained. The yeast extract–phosphate base medium was superior for isolation compared with blood and Sabouraud's agar. Yeast extract–phosphate with NH_4OH obtained 92% of the isolations; with gentamicin, 60%; with penicillin, streptomycin, and cycloheximide, 58%. In comparison, isolations made using blood agar amounted to 50%, and using Sabouraud's agar, 29%. The superiority of the yeast extract–phosphate medium with NH_4OH is attributed to its effectiveness in inhibiting the growth of saprophytic yeasts, bacteria, and fungi more than the other media.

Acknowledgments. Technical assistance was provided by Gisela Ramirez and Hugo Hempel.

References

1. Emmons CW, Binford CH, Utz JP: Histoplasmosis. Medical Mycology. Philadelphia, Lea and Febiger, 1963, pp 237–238
2. George LK, Ajello L, Gordon MA: A selective medium for the isolation of *Coccidioides immitis*. Science 114:387–389, 1951
3. Kapica L, Shaw CE, Bartlett GW: Inhibition of *Histoplasma capsulatum* by *Candida albicans* and other yeasts on Sabouraud's agar media. J Bacteriol 95:2171–2176, 1968
4. Larsh HW: Isolation and identification of *Histoplasma capsulatum*. Histoplasmosis. Edited by A Balows. Springfield, Ill., Charles C. Thomas, 1971, pp 271–276
5. Smith CD: Isolation and identification of *Histoplasma capsulatum* from soil. Histoplasmosis. Edited by A Balows. Springfield, Ill., Charles C. Thomas, 1971, pp 277–283